NITROGEN FIXATION IN LEGUMINOUS PLANTS. II.

IS SYMBIOTIC NITROGEN FIXATION INFLUENCED BY AZOTOBACTER?

By H. L. Jensen, Macleay Bacteriologist to the Society.
(*From the Department of Bacteriology, University of Sydney.*)

[Read 22nd July, 1942.]

Introduction.

Free-living nitrogen-fixing bacteria, especially *Azotobacter chroococcum*, have frequently been alleged to stimulate the growth of various higher plants (Jensen, 1942), and claims have been made that this also applies to leguminous plants. Joshi (1920) stated that this was the case with *Pisum sativum*, *Cicer arietinum* and *Phaseolus aconitifolius* grown in the absence of root-nodule bacteria. Heinze (1926) found that inoculation with *Azotobacter* in addition to root-nodule bacteria could increase the yields of peas, serradella and lupins in pot culture by as much as 40%. Heinze ascribed this to a stimulation of the nodule formation, but his guarded statements suggest that the effect was not constant, and neither his nor Joshi's data show whether the increase was statistically significant. Riede and Bucherer (1939) found the yield of soy beans in field trials somewhat increased by inoculation with *Azotobacter*, alone or in addition to *Rhizobium japonicum*; they give very few details, but the standard errors show that the increase was probably significant, especially in plants not inoculated with rhizobia. None of the authors have tried to explain the nature of this phenomenon which, as discussed elsewhere (Jensen, 1942), might be due to one or more of several causes:

1. Fixation of nitrogen by *Azotobacter*; this might be the case in the experiments of Joshi (1920), who added an unspecified amount of sugar to the sand medium. It has often been stated in a general way that *Azotobacter* may fix nitrogen at the expense of root secretion products from leguminous plants (Wilson, 1940), but proof of this is still lacking.

2. Plant development might be accelerated by growth-compounds elaborated by *Azotobacter*. We have comparatively little experimental evidence concerning the effect of different growth-compounds on intact leguminous plants. Chailakhian and Zhdanova (1938) stated that the growth of peas and vetches was depressed by pre-treatment of the seed with heteroauxin (α-indolacetic acid). Bonner and Greene (1938) found that thiamin (Vitamin B_1) promoted the development of certain slow-growing plants, but not of peas or beans. Pantothenic acid stimulates growth of lucerne seedlings in aseptic culture, according to McBurney *et al.* (1935), who regard the synthesis of panthothenic acid by the rhizobia as an important aspect of their symbiosis with the legumes. Laird and West (1937) observed that relatively high concentrations of "Bios 2b" (biotin?) induced abnormal growth of clover hypocotyles. Besides pure growth-compounds, some less defined organic substances, partly of microbial origin, have been stated to favour the growth of legumes in water- or sand-culture (Virtanen and v. Hausen, 1934; Niklewski and Wojciechowski, 1937). The existence of the bacterial symbiosis makes the influence of extraneously-supplied growth-compounds a more complicated problem than in non-legumes, since the rhizobia are capable, although to a very different degree, of synthesizing heteroauxin, thiamin, pantothenic acid, and biotin (Wilson, 1940). A positive effect might therefore be expected if the rate of synthesis of one or more of these growth-factors by either or both of the two symbionts were insufficient for optimal growth of the whole plant-rhizobia complex. There is also the possibility that *Azotobacter* might stimulate the growth of rhizobia outside the host plant and thus encourage nodule formation.

3. Seed germination might be favoured and the liability of the seedlings to attack by pathogenic organisms might be lessened by specific substances produced by *Azotobacter*.

Agricultural soils in Australia, even those of favourable reaction, are often deficient in *Azotobacter* (Jensen and Swaby, 1940). In connection with the now generally recognized need for leguminous crops as a counterbalance to nitrogen depletion, it thus seems desirable to investigate the possibility of improving the growth of legumes by some kind of *Azotobacter*-treatment as a supplement to inoculation of the seed with rhizobia. Some experiments have therefore been made with two of the most important pasture legumes: lucerne and white clover.

Experimental.

Agar cultures.—The first tests were carried out in big test-tubes with 20 or 40 c.c. of sterile agar medium ($CaHPO_4$ 0·1%, K_2HPO_4 0·02%, $MgSO_4$ 0·02%, NaCl 0·02%, $FeCl_3$ 0·01%, washed agar 0·6%; pH 6·5–6·6) and 2 or 3 plants, except in one case where 400-c.c. bottles with 80 c.c. agar and 4 plants were used. After disinfection with $HgCl_2$ and washing with sterile water, the seeds were sown in the agar and then inoculated with a pure culture of *Az. chroococcum*, or else soaked prior to sowing in a suspension of *Azotobacter*-cells; in this case the control seeds were soaked in sterile water. When the first true leaf had appeared (mostly after 7 to 10 days), the seedlings were inoculated with strains of *Rh. meliloti* or *trifolii*, which had been tested for effectiveness, and which grew very poorly or not at all in synthetic media, i.e., they appeared to have little capacity for synthesis of growth-compounds. In the first few trials 0·001% Na_2MoO_4 was added to the agar medium in order to make it optimal for *Azotobacter*. This was later omitted because other experiments had shown this concentration of molybdenum to be harmful to lucerne. In some experiments the inoculation with *Azotobacter* was replaced by addition to the agar of 2% sterile filtrate from 14-days-old cultures of *Azotobacter* in N-free mannite-solution. This filtrate stimulated markedly the growth of rhizobia in synthetic solution and appeared thus to be rich in biotin, the essential accessory growth-factor for rhizobia (Wilson, 1940). In another experiment, 10 or 50 p.p.m. of fresh cell-substance of *Azotobacter*, from 6-day cultures on N-free agar, were added directly to the agar medium. One experiment was carried out in liquid medium in order to eliminate the traces of growth-compounds which are regularly present in the agar; in this case the seeds rested on a thin pad of carefully boiled and washed cotton-wool supported by a few pieces of glass tubing. Each treatment comprised 4 to 8 replicates. The cultures were placed in a greenhouse and analysed for total nitrogen after 11 to 17 weeks' growth.

The rate of germination, the appearance of leaves and nodules, and the general vigour of growth were neither in lucerne nor in clover perceptibly influenced by *Azotobacter* or its culture-filtrate, alone or in addition to rhizobia. Growth of *Azotobacter* along the roots was not macroscopically visible, but tests on agar, and in some instances also direct microscopic examination, revealed its presence at the conclusion of the experiment in the agar cultures where it had been introduced. In the solution-cultures of lucerne it appeared to have died out. The analyses showed in no instance any significant influence of *Azotobacter* on the gain of nitrogen, as exemplified by the results of two typical experiments in Table 1. Addition of 50 p.p.m. fresh *Azotobacter*-substance seemed, although the evidence was not conclusive, to depress nitrogen-fixation in white clover.

TABLE 1.

Nitrogen Fixation by White Clover and Lucerne in Agar Culture.

Series.	Inoculum.	Replicates.	Total N per Culture, mgm.	
			Mean.	Standard Deviation.
White clover in test-tubes, plain agar, 105 days.	None.	4	0·23	±0·042
	Rhizobium.	6	1·69	±0·078
	Do. + *Azotobacter*.	5	1·65	±0·089
Same in agar + 2% *Azotobacter*-filtrate.	None.	4	0·23	±0·017
	Rhizobium.	6	1·59	±0·079
Lucerne in 400 c.c. flasks, 102 days.	None. 2 *Azotobacter*. 2	4	0·94	±0·097
	Rhizobium.	5	5·40	±0·651
	Do. + *Azotobacter*.	5	4·81	±0·334

Sand Cultures.—Since agar cultures permit only a limited growth under very artificial conditions, the experiments in this medium were supplemented with cultures of lucerne in practically nitrogen-free sand. In the first series, lucerne was grown in glazed earthenware pots each holding about 3 kgm. sand moistened with N-free Crone's solution; pots and sand were heated for 4 hours in steam before sowing. Disinfected seeds were sown, inoculated with *Rh. meliloti* and *Az. chroococcum*, and thinned to 7 plants per pot. Another experiment was carried out in wide-necked 500-c.c. glass bottles holding 500 gm. of sand with the same solution, and 2 plants per bottle. The cultures were placed in a greenhouse and watered with sterile water. In both series the lucerne grew rather poorly, and no influence of the *Azotobacter*-treatment was noticeable, although *Azotobacter* at the end of the experiment was found in the cultures where it had been introduced, but not in the others. The agreement between replicates, moreover, was unsatisfactory; a third experiment was therefore made in pots with sand moistened with a solution corresponding to the agar medium. This time the growth was excellent and the agreement between replicates much better, but again there was no significant effect of *Azotobacter*. The results of the sand-culture experiments are seen in Table 2.

TABLE 2.
Influence of Azotobacter *on Nitrogen Fixation by Lucerne in Sand-Culture.*

Series.	Inoculum.	Replicates.	Dry Matter per Pot, gm.		N in Dry Matter, %.
			Mean.	Standard Deviation.	
I. Pot cultures.	None.	4	0·10	±0·026	1·29
29/7/40–30/12/40.	*Azotobacter.*	3*	0·12	±0·017	1·22
Whole plants.	*Rhizobium.*	7	0·81	±0·520	2·14
	Do. + *Azotobacter.*	6	0·73	±0·238	2·15
II. Flask cultures.	*Rhizobium.*	6	0·25	±0·154	
27/2/41–16/10/41.	Do. + *Azotobacter.*	6	0·32	±0·100	
Tops only.					
III. Pot cultures.	*Rhizobium.*	3	Tops: 4·18	±0·308	3·93
4/4/41–13/8/41.			Roots: 1·81	±0·217	3·31
Tops and roots.	Do. + *Azotobacter.*	3	Tops: 4·07	±0·135	4·06
			Roots: 1·54	±0·136	3·52

* Two pots lost by infection with *Rhizobium.*

Influence of Straw on the Growth of Lucerne.—The negative results of the sand-culture experiments might be due to failure of *Azotobacter* to grow well in the pure sand, where the plant roots represent the only source of organic nutrients. It has sometimes been observed (Thornton, 1929), that addition of straw to the soil favours nodule formation and thus improves the growth of leguminous plants; this phenomenon might conceivably be partly due to a stimulating influence of *Azotobacter*, which under suitable soil conditions will multiply vigorously when fresh straw is added. A pot experiment was therefore carried out with lucerne in sand + N-free Crone's solution, with and without addition of 0·5% finely ground wheat straw. Five replicates were run, with 12 plants in each; two extra pots with addition of straw were left unplanted. The medium was not sterilized, but inoculated with a small amount of soil suspension rich in *Azotobacter*, in addition to inoculation with *Rh. meliloti*. The first crop of lucerne was almost completely destroyed by the addition of straw, and in the control pots the growth was also somewhat poor and irregular, as shown in Table 3. A second crop of lucerne was therefore sown.

At the time of sowing the second crop, *Azotobacter* had developed richly in the sand + straw, where plate counts showed a number of 47,000 per gm. against 8 per gm. in the straw-free sand. At the same time the sand + straw had become greatly enriched with the growth-factor for *Rhizobium*, as shown by a tentative assay: portions of original sand, as well as sand from the unplanted pots with straw, were extracted, first with cold water for one hour, then with hot water by autoclaving 15 min. at 20 lb. pressure. The filtrates were then condensed on a water-bath to such a volume that 1 c.c. represented

TABLE 3.
Influence of Straw on the Growth of Lucerne.

	Straw Added, %.	Replicates No.	Dry Matter per Pot, gm. Mean.	Standard Deviation.
1st Crop: 25/10/40–20/3/41.	0	5	0·47	±0·554
Whole plants weighed.	0·5	5	0·10	±0·054
2nd Crop: 20/3/41–13/8/41. Tops and roots weighed separately.	0	5	Tops: 2·22	±0·375
			Roots: 2·76	±0·603
	0·5	5	Tops: 1·31	±0·265
			Roots: 1·32	±0·284
$NaNO_3$ added. 6/5/41–8/8/41.	0	3	1·96	±0·144
Whole plants weighed.	0·5	3	2·00	±0·086

the extract from 8 gm. of sand. Varying amounts of the extracts were then added to a synthetic nutrient solution (mannite, $NaNO_3$, mineral salts) in which *Rh. meliloti* was grown for 6 days at 28–30°C., when the bacterial substance was removed by centrifugation, dried and weighed. The following results (averages of duplicate determinations) were obtained:

Addition to synthetic solution.	Bacterial substance, mgm. per 25 c.c. of medium with Cold-water extract.	Hot-water extract.
Nothing (control)	0·1	0·2
1% extract of pure sand	0·8	0·9
5% ,, ,, ,, ,, ,, ...	2·2	2·8
1% ,, ,, sand+straw	1·6	1·3
5% ,, ,, ,, ,, ...	4·7	4·6

Control experiments in solutions without mannite or nitrate showed that the stimulating effect was not due simply to the extracts providing assimilable carbon or nitrogen. The sand itself thus contains a certain amount of the growth-factor (presumably biotin), but its concentration is considerably increased by the addition of straw. Nevertheless the depressing influence of straw on the growth of lucerne is still quite distinct in the second crop (Table 3). A control experiment, also included in Table 3, shows that the straw has no such effect in the presence of combined nitrogen.*

Since the harmful effect of the straw might be due to increased fungal attack on the seedlings in the early stage of growth before nitrogen fixation becomes active, a few supplementary experiments were carried out under biologically-controlled conditions. Lucerne plants were grown from disinfected seeds in 500-c.c. flasks with 200 gm. of sand and 50 c.c. of mineral solution plus hot-water extract of wheat straw† in a quantity equivalent to the soluble constituents of 1·0 gm. straw per flask. The culture-flasks were plugged with cotton-wool and sterilized in the autoclave. Some cultures were inoculated with *Rh. meliloti* and *Az. chroococcum*, others were left uninoculated but given 0·1% $NaNO_3$ in the nutrient solution. The results (Table 4) show that the sterile straw-extract is not *per se* harmful to the plants, but becomes so in the presence of *Rh. meliloti*, and this effect appears to be intensified by *Azotobacter*, which multiplies strongly in the presence of straw-extract.

Other experiments showed that straw-extract in which *Rhizobium* or *Azotobacter* had grown for 5 weeks at 28–30°C. had an inhibitory effect on the roots of lucerne

* Cf. Collison and Conn (1925), who found straw-extract harmful to barley seedlings in water- or sand-culture but not in soil.

† Prepared by autoclaving finely chopped wheat straw with 10 times its quantity of tap-water, and filtering.

TABLE 4.
Influence of Straw-extract on Growth of Lucerne in Sterile Sand.

	Inoculum.	Straw-extract, %.	Dry Weight of Plants, gm.	*Azotobacter* per gm. of Sand at End of Exp.
Sand − $NaNO_3$. 31/3/41–8/8/41.	*Rh. meliloti.*	0	0·22	0
	Do. + *Azotobacter.*	0	0·21	860
	Rh. meliloti.	5	0·15	0
	Do. + *Azotobacter.*	5	0·12	>250,000
Sand + $NaNO_3$. 7/5/41–8/8/41.	None.	0	0·17	
	Do.	5	0·16	

seedlings. Duplicate lots of 50 disinfected lucerne seeds were allowed to germinate for 5 days at 24–25°C. in Petri dishes with sterile filter-paper moistened with (1) sterile tap-water as control, (2) plain sterile straw-extract, and (3) extracts in which the two bacteria had grown; these extracts were restored to original volume, the bacteria were removed by sharp centrifugation, and the liquid was used without any further treatment as well as after sterilization in the autoclave. After 5 days the length of stems and roots was measured. The percentage of germination and the lengths of the stems were not significantly affected by any treatment. Table 5 gives the mean lengths, with standard errors, of the roots. The plain straw-extract is inactive, except perhaps in extra high concentration, but the retarding effect of the culture solutions is quite marked and is not diminished by autoclaving. In agar cultures inoculated with rhizobia the plain extract as well as the culture solutions caused shortening and thickening of young lucerne and clover roots. The phenomenon was much like that observed by Collison and Conn (1925) in barley seedlings, except that it does not appear to be caused by substances originally present in the straw-extract, but to arise under the influence of bacteria. It seems probable that the active substance is a heteroauxin; a very pronounced stunting and thickening of the roots of white clover and especially lucerne was seen in agar with synthetic heteroauxin (α-naphtyl-acetic acid) in concentrations as low as 10^{-7} to 10^{-8}. This malformation of the roots may result in diminished nodule formation, which would explain the apparent harmlessness of the straw in the presence of nitrate.*

TABLE 5.
Influence of Straw-extract on Early Growth of Lucerne Seedlings.

I. Plain Straw-extract.				II. Filtrate from *Rh. meliloti.*			
Conc. of Extract, %.		Germination, %.	Length of Roots, mm.	Conc. of Extract, %.		Germination, %.	Length of Roots, mm.
0	(*a*)	80	26·3±1·54	0	(*a*)	84	28·9±1·50
	(*b*)	82	26·2±1·83		(*b*)	82	28·3±1·29
10	(*a*)	80	27·8±1·44	10	(*a*)	80	20·1±0·76
	(*b*)	86	25·1±1·26	(Untreated)	(*b*)	70	20·1±1·31
50	(*a*)	80	22·3±0·95	10	(*a*)	80	21·7±0·85
	(*b*)	90	27·7±1·20	(Autoclaved)	(*b*)	70	21·9±1·05
III. Filtrate from *Az. chroococcum*, Untreated.				IV. Filtrate from *Az. chroococcum*, Autoclaved.			
0	(*a*)	82	24·6±1·13	0	(*a*)	76	28·4±1·38
	(*b*)	78	26·3±1·51		(*b*)	78	31·3±1·64
5	(*a*)	70	17·0±0·97	5	(*a*)	72	21·2±1·22
	(*b*)	84	19·9±1·14		(*b*)	80	17·0±1·01
20	(*a*)	80	15·3±1·04	20	(*a*)	66	16·2±1·08
	(*b*)	84	17·0±1·17		(*b*)	72	16·5±1·43

* Cf. also the observation of Laird and West (1937) that high doses of biotin suppress temporarily the formation of root-hairs, through which the infection with rhizobia takes place.

Several other germination experiments with different kinds of seed showed that treatment with cell suspensions or culture-filtrate of *Azotobacter* had no significant influence on the rate or percentage of germination, no matter whether this was high (*Medicago sativa, Trifolium pratense*) or low (*Medicago denticulata, Trifolium glomeratum*).

The Growth of Azotobacter *in the Rhizosphere of Lucerne and Clover.*—It has often been stated that the rhizosphere of leguminous plants may offer a favourable habitat for *Azotobacter* (Wilson, 1940), but quantitative data in support thereof are still lacking. Some such observations have therefore been made on lucerne and subterranean clover, which were grown in almost N-free sand of faintly alkaline reaction, supplied with mineral nutrients and inoculated with *Az. chroococcum* as well as rhizobia. A number of plants were dug up at successive stages of growth, and counts of *Azotobacter* were made in the sand as well as the rhizosphere (roots with adhering sand) by plating on N-free dextrine agar. A few supplementary counts of the general rhizosphere-flora were made on soil-extract mannite agar. Table 6 gives the results, which show that the numbers of *Azotobacter* in the sand supporting plant growth are not appreciably higher than those in the unplanted control sand. In the actual rhizosphere of both plants there is a discernible, but not very striking, increase in *Azotobacter*, which, however, accounts for only an infinitesimal fraction of the total rhizospheral flora, as shown by the counts on soil-extract agar.

TABLE 6.
Numbers of Azotobacter *in the Rhizosphere of Lucerne and Clover.*

Incubation.	Numbers of *Azotobacter* per gm.				
	Control Sand.	Lucerne.		Clover.	
		Sand.	Roots.	Sand.	Roots.
Start	450				
43 days	600	230	660	460	900
63 ,, ...	450	520	1,000	240	680
90 ,, ...	140	460	940	420	1,550
		Lucerne roots.		Clover roots.	
"Total" counts of bacteria, million per gm.	43 d.	103·7		69·6	
	63 d.	75·6		50·4	

Some 10 to 15% of the colonies on this medium belonged to a clearly-recognizable type resembling the root-nodule bacteria: small, irregular, spore-free rods forming big, smooth, soft colonies, very fluid and semi-transparent from the clover roots, more opaque and whitish from the lucerne roots. Nine isolates from the latter actually proved to be *Rh. meliloti*, as shown by their production of nodules on lucerne seedlings. The rhizobia would thus appear to be much more important inhabitants of the rhizosphere than *Azotobacter*.

Since the influence on the numbers of *Azotobacter* was so small, an experiment was performed to see whether the lucerne roots do at all secrete organic substances serviceable for *Azotobacter*. Lucerne, inoculated with effective rhizobia, was grown for 16 weeks in 1-pint bottles with 150 c.c. of agar and 6 plants in each; after removal of the plants the portions of agar were combined, melted, given small additions of Na_2MoO_4 and $CaCO_3$ and distributed in lots of 100 c.c. to 500-c.c. Erlenmeyer flasks, which were sterilized, inoculated with *Az. chroococcum*, and incubated for 14 days at 28–30°C. No visible growth took place, and no nitrogen was fixed, while a control culture showed that the medium was favourable except for lack of available carbon:

	Mgm. N per flask.
Sterile controls (triplicate)	0·58
Azotobacter-cultures (triplicate)	0·59
Do. + 0·2 gm. mannite (single)	2·33

Intact roots thus do not, at least under these conditions, secrete nutrients for *Azotobacter* in any discernible quantity. The root-substance itself, on the other hand, is comparatively rich in such compounds. Dried and finely ground roots of 20-weeks-old lucerne plants grown in sand culture were extracted with boiling water, small quantities of $FeCl_3$, $CaCO_3$ and Na_2MoO_4 were added to the extract, and *Az. chroococcum* was grown in this medium for 12 days at 28°C. Good growth, but only a moderate nitrogen fixation, took place; the following quantities of nitrogen were found per 25 c.c. of medium containing 0·348 gm. dry matter (excluding the added salts) which represented the hot-water soluble constituents of 0·75 gm. air-dry root material:

Sterile control before incubation (duplicate)	7·76 mgm.
Do. after incubation (single)	7·74 ,,
Azotobacter-culture (duplicate)	8·24 ,,

The small increase in *Azotobacter* in the rhizospheres (Table 6) is presumably due to such soluble matter together with decomposition products of sloughed-off root particles. But since young root tissue is comparatively rich in nitrogen, a good deal of which is soluble, *Azotobacter* is badly placed in the competition with non-nitrogen-fixing organisms and only reaches relatively low numbers from which no significant nitrogen fixation can be expected. That this is actually the case was shown by another experiment: 0·5% finely ground, air-dry root-material of lucerne, containing 2·06% N, was added to a mixture of loamy soil and sand, of pH 7·4 and well supplied with mineral nutrients, including molybdenum. Duplicate portions of moderately moist soil were incubated for 4 weeks at 28°C., periodical plate counts of *Azotobacter* were made, and total as well as mineral nitrogen was determined before and after incubation. Control soil without root-material showed no significant changes in *Azotobacter* or mineral nitrogen. The data on soil + roots are given in Table 7, and show that *Azotobacter* indeed multiplies to an extent only rarely observed under field conditions, but this is accompanied by a small, although significant, loss of total nitrogen. Nitrification of the added organic matter is already noticeable after 4 weeks, and gains of nitrogen during subsequent stages of the decomposition-process can therefore be regarded as out of question.

TABLE 7.
Numbers of Azotobacter *and Nitrogen Content in Soil+Lucerne Roots.*

	Incubation, days.			
	0	8	18	28
Azotobacter per gm. dry soil ..	(*a*) 33	5,860	7,580	10,100
	(*b*) 33	10,400	6,260	15,100

	Before incubation.	After incubation.
Total N, p.p.m., mean and standard error.*	229±0·50	(*a*) 220±0·54 (*b*) 220±1·88
$(NH_4+NO_3)N$, p.p.m.	13·2	(*a*) 15·9 (*b*) 16·8

* Initial N-determination based on 4, final ones on 6 and 7 replicates.

Although it is possible that other species of leguminous plants may behave differently, the activity of *Azotobacter* thus seems most unlikely to be a source of serious error in experiments on nitrogenous excretion by the roots of legumes (Wilson, 1940).

Discussion.

The experiments have shown quite consistently that presence of *Azotobacter* in the growth-medium has no perceptible stimulating effect on the growth of lucerne or clover, in whose rhizosphere it grows so sparsely that benefit to the plants through nitrogen fixation may be regarded as out of question, and an effect of its growth-hormones (even assuming that the plants were deficient in growth-compounds) also appears problematical, since the ability to synthesize such substances is shared by many other micro-organisms. On the other hand, excessive numbers of *Azotobacter*, produced by

extra supply of nutrients, were actually harmful to the plants and especially to the development of roots, as was also found by Krasilnikov and Garkina (1939), who ascribed this effect to specific metabolic products rather than growth-compounds. An uncritical application of straw or similar materials to leguminous crops in soil very poor in nitrogen should therefore be avoided.

These conclusions, of course, apply only to the direct influence of *Azotobacter chroococcum* on the plants from the seedling stage and onwards, under laboratory or greenhouse conditions. The possibilities of improved seed germination and of antibiotic effects against plant pathogens still remain, and it is conceivable that the apparent benefit to soy beans in the experiments of Riede and Bucherer (1939) may find its explanation herein.

Summary.

Lucerne and white clover were grown in agar or sand-culture with or without inoculation with *Azotobacter chroococcum* in addition to root-nodule bacteria. In no case could any significant stimulation of the nitrogen fixation by *Azotobacter* be observed.

Addition of straw to the sand increased the numbers of *Azotobacter* greatly, but had a detrimental effect on lucerne. The same was the case with straw-extract inoculated with pure cultures of *Azotobacter*. Filtrates from such cultures inhibited the elongation of roots of young lucerne seedlings. No harmful effect of straw was observed in the presence of combined nitrogen.

In the rhizosphere of lucerne and clover grown in sand, the numbers of *Azotobacter* were only moderately increased in comparison with the sand-medium itself. Intact roots of lucerne did not appear to excrete organic substances that support growth of *Azotobacter*.

Acknowledgement.

The collaboration of Mr. R. C. Betty, M.Sc., who has carried out much of the analytical work, is gratefully acknowledged.

References.

Bonner, J., and Greene, J., 1938.—Vitamin B_1 and the Growth of Green Plants. *Bot. Gaz.*, 100, 226-237.

Chailakhyan, M. C., and Zhdanova, L. P., 1938.—Vliyanie geteroauksina na rost i razvitiye rastenii pri obrabotka semyan. *Bull. Acad. Sci. URSS., Ser. Biol.*, 1938, No. 5-6, 1281-1296.

Collison, R. C., and Conn, H. J., 1925.—The Effect of Straw on Plant Growth. *N.Y. Agric. Exp. Stat. Techn. Bull.* 114.

Heinze, B., 1926.—Mikrobiologische Untersuchungen. *Landw. Jahrb.*, 64, 127-169.

Jensen, H. L., 1942.—Bacterial Treatment of Non-leguminous Seeds as an Agricultural Practice. *Aust. J. Sci.*, 4, 117-120.

————, and Swaby, R. J., 1940.—Further Investigations on Nitrogen-fixing Bacteria in Soil. Proc. Linn. Soc. N.S.W., 65, 557-564.

Joshi, N. V., 1920.—Studies on the Root Nodule Organisms of the Leguminous Plants. *Mem. Dept. Agric. Ind., Bact. Ser.*, 1, 247-276.

Krasilnikov, N. A., and Garkina, N. R., 1939.—Vliyanie bakterii na rost isolirovannykh kornei rastenii. *Mikrobiologiya*, 8, 952-958.

Laird, D. G., and West, P. M., 1937.—The Influence of Bios on Nodule Bacteria and Legumes. *Canad. J. Res.*, 15C, 1-6.

McBurney, C. H., Bollen, W. B., and Williams, R. J., 1935.—Pantothenic Acid and the Nodule Bacteria-Legume Symbiosis *Proc. Nat. Acad. Sci. Wash.*, 21, 301-304.

Niklewski, B., and Wojciechowski, J., 1937.—Über den Einfluss der wasserlöslichen Humusstoffe auf die Entwickelung einiger Kulturpflanzen. *Bodenk. u. Pflanzenern.*, 4 (49), 294-327.

Riede, W., and Bucherer, H., 1939.—Über Lebensdauer, Wirksamkeit und Leistung der Sojaknöllchenbakterien. *Cent. Bakt.*, II, 100, 25-34.

Thornton, H. G., 1929.—The Effect of Fresh Straw on the Growth of Certain Legumes. *J. Agric. Sci.*, 19, 563-572.

Virtanen, A. I., and v. Hausen, S., 1934.—Effect of Yeast Extract on the Growth of Plants. *Nature*, 133, 383.

Wilson, P. W., 1940.—The Biochemistry of Symbiotic Nitrogen Fixation. Madison.